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Effect of the Proton Electrochemical Gradient on Maleimide Inactivation of Active Transport in *Escherichia coli* Membrane Vesicles†

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ABSTRACT: Many active transport systems present in *Escherichia coli* membrane vesicles are inhibited by maleimides and other sulfhydryl reagents. These reagents do not interfere with the oxidation of reduced phenazine methosulfate or with the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$). The rate of inactivation of the β-galactoside transport system by various maleimides is increased in the presence of reduced phenazine methosulfate, and it is shown that the electrochemical proton gradient is responsible for the effect. Furthermore, similar effects are observed with the proline and melibiose transport systems. Thus, it appears that either the reactivity or accessibility of a sulfhydryl group(s) in each of these carriers is altered by the presence of a transmembrane $\Delta\bar{\mu}_{H^+}$. The findings are consistent with the notion that $\Delta\bar{\mu}_{H^+}$, in addition to acting as the immediate driving force for active transport, may bring about structural or conformational changes in certain membrane proteins that catalyze active transport.

The chemiosmotic hypothesis of Mitchell (1961, 1966, 1968, 1973, 1979) proposes that energy derived from respiration is transformed into a transmembrane electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$)[1] that is the immediate driving force for many active transport systems in bacterial cells. Accordingly, respiration-dependent accumulation of substrates is postulated to result from the obligatory coupling of substrate translocation with proton translocation mediated by substrate-specific membrane proteins (carriers or porters).

Cytoplasmic membrane vesicles prepared from *Escherichia coli* by osmotic lysis (Kaback, 1971; Short et al., 1975) have the same polarity of the membrane as the intact cell (Owen & Kaback, 1978, 1979a,b) and retain the capacity to catalyze the active transport or many substrates (Kaback, 1974a; Ramos et al., 1976). Studies performed both with intact cells (Harold, 1976) and with membrane vesicles (Ramos & Kaback, 1977a-c; Konings & Boonstra, 1977) have provided virtually unequivocal evidence for the central role of chemiosmotic phenomena in respiration-dependent active transport.

Kinetic studies of lactose transport in *E. coli* membrane vesicles have provided some interesting insights into the effects

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[1] Abbreviations used: DTT, dithiothreitol; PMS, phenazine methosulfate; TMG, methyl 1-thio-β-D-galactopyranoside; NNM, *N*-(2-naphthyl)maleimide; MBTA, (4-(*N*-maleimido)benzyl)trimethylammonium iodide; GSMal, glutathione maleimide; NEM, *N*-ethylmaleimide; pBM, *N*-(*p*-benzoyl)maleimide; TSM, *N*-(*p*-tolylsulfonyl)-maleimide; $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; ΔpH, pH gradient; $\Delta\Psi$, membrane potential.

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of $\Delta\bar{\mu}_{H^+}$ on the overall translocation reactions. A comparison of active transport and facilitated diffusion of lactose shows that $\Delta\bar{\mu}_{H^+}$ leads to a dramatic decrease in the apparent K_m (Kaczorowski et al., 1979; Wright et al., 1979). A more recent study of the effects of $\Delta\bar{\mu}_{H^+}$ on the kinetics of lactose transport (Robertson et al., 1980) demonstrates that the β-galactoside transport system catalyzes two distinctly different reactions, one exhibiting a high apparent K_m and the other a much lower apparent K_m, and the interconversion of the low and the high K_m reactions is dependent on the magnitude of $\Delta\bar{\mu}_{H^+}$ to the second power. In order to explain the observations, it was tentatively suggested that $\Delta\bar{\mu}_{H^+}$ causes a structural change in the *lac* carrier protein; however, it was emphasized that a change in the rate-limiting step for transport might account for the findings without a structural alteration in the *lac* carrier.

Despite widespread agreement on the chemiosmotic nature of active transport, little information is available regarding the mechanism of the phenomenon, particularly the chemical reactions and/or functional groups involved and whether or not they are affected directly by $\Delta\bar{\mu}_{H^+}$. It was recently reported that lactose transport in *E. coli* membrane vesicles is inhibited by diethyl pyrocarbonate (Padan et al., 1979). Moreover, inhibition of transport by this reagent is accelerated by $\Delta\bar{\mu}_{H^+}$, implying that the reactivity or accessibility of a functionally required histidinyl group(s) is altered. Similarly, in mitochondria, it has been demonstrated that the inhibition of phosphate (LeQuoc et al., 1977) and adenine nucleotide diphosphate (LeQuoc et al., 1979) translocation by certain sulfhydryl reagents is accelerated by $\Delta\bar{\mu}_{H^+}$.

Several carriers present in *E. coli* membrane vesicles are inactivated by sulfhydryl reagents (Kepes, 1960; Fox & Kennedy, 1965; Barnes & Kaback, 1971; Kaback & Barnes, 1971; Patel & Kaback, 1978). In this report, we examine the effect of energization of the membrane on inactivation of several transport systems by different maleimides. The results show that $\Delta\bar{\mu}_{H^+}$ brings about changes in the reactivity of sensitive sulfhydryl groups in the transport systems investigated. Such effects are consistent with the involvement of conformational or structural changes in the carriers in response to $\Delta\bar{\mu}_{H^+}$.

Materials and Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$), which is constitutive for β-galactoside transport and lacks the melibiose transport system, was grown on minimal salts medium (Davis & Mingioli, 1950) containing 1.0% disodium succinate (hexahydrate), and membrane vesicles were prepared by osmotic lysis as described (Kaback, 1971; Short et al., 1975). *E. coli* W3113-2 (lac z^-y^-, mel A^+B^+), which has a deletion for the *lac y* gene and is inducible for the melibiose transport system (Lopilato et al., 1978), was a generous gift from Dr. T. H. Wilson, Harvard University Medical School. These cells were grown in medium 63 (Cohen & Rickenberg, 1956) supplemented with 1% pancreatic digest of casein (Bacto-tryptone, Difco), 0.5 mg/L thiamin, and 10 mM melibiose, and vesicles were prepared as described (Cohn & Kaback, 1980). All vesicles were stored in 0.1 M potassium phosphate (pH 6.6) and frozen in liquid nitrogen.

For experiments at various pHs, vesicles were thawed and resuspended in at least a 50-fold excess of the desired buffer. After standing at room temperature for 30 min, the vesicles were collected by centrifugation (40000*g* for 30 min), washed once, and resuspended in the same buffer to the desired protein concentration.

Treatment with Maleimides. Vesicles were treated with maleimides by two different methods. In the first method, the treatment was carried out by adding a given maleimide to a 50-μL reaction mixture containing, in final concentrations, membrane vesicles at 2 mg of membrane protein/mL, 50 mM potassium phosphate (at the desired pH), and 10 mM magnesium sulfate. The reaction was stopped by addition of excess dithiothreitol (DTT), after which active transport was assayed as described below. For treatment with maleimides in the presence of reduced phenazine methosulfate (PMS), the reaction mixture was the same except that 20 mM potassium ascorbate (at the desired pH) and 0.1 mM PMS were added 30 s before the maleimide, and the reaction was carried out under an atmosphere of water-saturated oxygen (Kaback, 1974b). The reaction was again stopped by addition of DTT, and active transport was assayed immediately in situ as described below.

The second method involved addition of the maleimide to a larger reaction mixture (1–2 mL total volume) containing membrane vesicles at a concentration of 1.0 mg of membrane protein/mL in 0.1 M potassium phosphate (pH 6.6). As above, potassium ascorbate and PMS were added 30 s before the maleimide where indicated. The reaction was stopped by addition of a large volume (40 mL) of ice-cold buffer containing DTT. The vesicles were collected by centrifugation, washed, and finally resuspended in buffer suitable for determining active transport.

Transport Assays. Uptake of radiolabeled substrates into membrane vesicles in the presence of ascorbate and PMS was determined by filtration essentially as described (Kaback, 1974b). In all cases, ascorbate and PMS were added to the vesicles at least 30 s before the substrate in order to assure complete energization of the vesicles before uptake was initiated. Following addition of the labeled substrate, uptake was allowed to proceed for the times indicated, over which the uptake was linear, before termination of the reaction and filtration on nitrocellulose filters. All values reported for substrate accumulation are initial rates.

Protein. Protein was measured according to Lowry et al. (1951) by using bovine serum albumin as standard.

Materials. [1-^{14}C]Lactose was purchased from Amersham/Searle, and [U-^{14}C]proline and [*methyl*-^{14}C]methyl 1-thio-β-D-galactopyranoside (TMG) were purchased from New England Nuclear. *N*-(2-Naphthyl)maleimide (NNM) was obtained from Dr. G. Ailhaud, University of Nice, Nice, France, (4-(*N*-maleimido)benzyl)trimethylammonium iodide (MBTA) was a generous gift of Dr. A. Karlin, Columbia University, College of Physicians and Surgeons, *N*-(*p*-benzoyl)maleimide (pBM) was obtained from Dr. G. Carvajal, Polytechnic Institute, Mexico City, and glutathione maleimide (GSMal) was donated by Drs. David Schachter and Richard Abbott, Columbia University, College of Physicians and Surgeons. *N*-Ethylmaleimide (NEM) (gold label) was purchased from Aldrich, and *N*-(*p*-tolylsulfonyl)maleimide (TSM) was from the Alfred Bader Library of Rare Chemicals (Aldrich). Nigericin was generously provided by Dr. John Wesley, Hoffmann-LaRoche, Inc., and valinomycin was obtained from Calbiochem. All other chemicals were of reagent grade and from commercial sources.

Results

The β-galactoside transport system of *E. coli* is inactivated by sulfhydryl reagents (Kepes, 1960; Fox & Kennedy, 1965; Barnes & Kaback, 1971; Kaback & Barnes, 1971), and this property has been used to covalently label and identify the *lac* carrier protein (Fox & Kennedy, 1965). For the studies re-

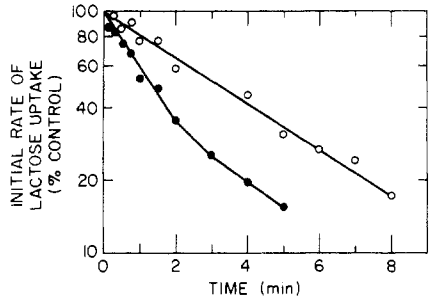

FIGURE 1: Time course of inhibition of lactose transport by NEM in the presence and absence of reduced PMS. Aliquots of *E. coli* ML 308-225 membrane vesicles (0.1 mg of membrane protein) were diluted to a final volume of 50 μL containing, in final concentrations, 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate. For inhibition in the absence of an energy source (○), NEM was added to a final concentration of 0.5 mM, and the mixture was incubated at 26 °C for the times indicated, after which 2.0 mM DTT was added to stop the reaction. Uptake was terminated after 10 s by addition of 2 mL of 50 mM potassium phosphate (pH 5.5) and 100 mM lithium chloride and immediate filtration on nitrocellulose filters (0.45-μm pore size). The filter was washed with another 2 mL of the termination buffer. For inhibition by NEM in the presence of energy (●), the vesicles were incubated for 30 s under oxygen with added PMS and ascorbate, following which 0.5 mM NEM (final concentration) was added, and the incubation was continued for the times indicated. The inhibition reaction was stopped by addition of DTT, followed immediately by 0.4 mM [^{14}C]lactose. After 10 s, the uptake reaction was terminated as above. Zero times of inhibition were carried out as above but with the DTT added before the addition of NEM. The uninhibited rate (100% activity) was 58 nmol of lactose min^{-1} (mg of protein)$^{-1}$.

Table I: Kinetics of Inhibition of Lactose Active Transport in the Presence and Absence of Energy by a Variety of Maleimides[a]

maleimide	$t_{1/2}$ of inhibition (min) −PMS	$t_{1/2}$ of inhibition (min) +PMS	$t_{1/2}$(−PMS)/ $t_{1/2}$(+PMS)
NEM (0.5 mM)	3.2	1.3	2.5
NNM (20 μM)	4.0	1.5	2.7
MBTA$^+$ (100 μM)	2.0	0.75	2.7
pBM$^-$ (40 μM)	6.8	2.8	2.4
TSM (200 μM)	10.0	3.7	2.7
GSMal (2.9 mM)	6.9	2.7	2.6

[a] *E. coli* ML 308-225 membrane vesicles were treated with the indicated maleimides (final concentrations in parentheses) in the presence and absence of PMS plus ascorbate for various times, following which initial rates of active lactose uptake were determined as described in Figure 1. $t_{1/2}$ denotes the time required for 50% inactivation of the initial rate of lactose uptake (see Figure 1).

ported here, it is important to note that in *E. coli* membrane vesicles sulfhydryl reagents have no effect on the oxidation of the reduced PMS or on the $\Delta\bar{\mu}_{H^+}$ generated in the presence of this electron donor (Patel & Kaback, 1978). Thus, we have used the initial rate of lactose uptake driven by reduced PMS as a measure of active *lac* carrier following treatment with various maleimides. The rate of inhibition of respiration-driven lactose uptake at a fixed concentration of NEM is approximately first order (Figure 1). Interestingly, when the same reaction is carried out in the presence of reduced PMS, the rate of inhibition by NEM is increased by a factor of about 2.5.

Similar experiments have been carried out with a variety of other maleimides (Table I). These include a very hydrophobic maleimide (NNM), maleimides that are charged (pBM$^-$, MBTA$^+$), and a hydrophilic, impermeant maleimide (GSMal) that is the monoadduct of glutathione with bis(maleidomethyl) ether (Abbott & Schachter, 1976). Although the concentrations required to inhibit lactose uptake vary considerably from one maleimide to another, in each case the rate of inhibition is increased (i.e., $t_{1/2}$ is decreased) in the presence of reduced PMS. In view of the diversity of the maleimides examined (size, hydrophobicity, and charge), it is particularly interesting that reduced PMS enhances the rate of inhibition by essentially the same factor (2.5–3-fold) in each case.

Table II: pH Dependence of NEM Inhibition of Lactose Transport in the Presence and Absence of Energy[a]

pH	$t_{1/2}$ of inhibition (min) −PMS	$t_{1/2}$ of inhibition (min) +PMS	$t_{1/2}$(−PMS)/ $t_{1/2}$(+PMS)
5.5	4.8	4.5	1.1
6.6	3.2	1.3	2.5
7.5	1.1	0.65	1.7

[a] Membrane vesicles of *E. coli* ML 308-225 were diluted, washed, and resuspended to 2.0 mg of membrane protein/mL in buffer containing 50 mM potassium phosphate and 10 mM magnesium sulfate at the indicated pH. At each pH, a time course of inhibition of active lactose uptake by 0.5 mM NEM was determined in the presence and absence of reduced PMS as described in Figure 1. Uninhibited rates of lactose uptake were 20, 55, and 51 nmol of lactose min^{-1} (mg of protein)$^{-1}$ at pH 5.5, 6.6, and 7.5, respectively.

The rate of inhibition of lactose uptake by NEM is pH dependent (Table II), becoming more rapid at alkaline pH. However, the degree to which the rate of inhibition is accelerated by reduced PMS appears to have an optimum, being somewhat more pronounced at pH 6.6 than at pH 7.5 and almost absent at pH 5.5. This pH profile is superficially similar to the pH profile for the initial rate of lactose uptake in the presence of reduced PMS (Robertson et al., 1980, and unpublished observations).

Strain W3113-2 of *E. coli* has a genetic deletion for lactose transport and a temperature-stable, inducible transport system for melibiose (Lopilato et al., 1978). When this transport system is assayed with methyl 1-thio-β-D-galactopyranoside (TMG) as substrate, the system is strictly dependent on the presence of sodium (or lithium), and the uptake of TMG occurs in symport with sodium (Lopilato et al., 1978; Cohn & Kaback, 1980). The melibiose transport system of *E. coli*, like the β-galactoside transport system, is inhibited by sulfhydryl reagents (Lopilato et al., 1978; Cohn & Kaback, 1980), and substrates of the melibiose carrier partially protect against inhibition by NEM. As shown in Figure 2, the $t_{1/2}$ of inhibition by 50 μM NEM is increased from 4 to 11 min in the presence of 20 mM melibiose and 10 mM sodium ion, indicating that the inhibition occurs at the carrier level. Furthermore, as with the *lac* carrier, reduced PMS increases the rate of inactivation by NEM (Figure 2). With 50 μM NEM, half-times of inhibition of 4 min and 0.8 min are observed in the absence and presence of reduced PMS, respectively.

For determination of whether or not the effect of reduced PMS is a consequence of the generation of $\Delta\bar{\mu}_{H^+}$, *E. coli* W3113-2 vesicles were incubated with NEM in the presence and absence of reduced PMS and dinitrophenol. Dinitrophenol abolishes $\Delta\bar{\mu}_{H^+}$ but has no effect on the oxidation of the reduced PMS. As shown in Table III, dinitrophenol completely eliminates the stimulatory effect of the electron donor on the rate of NEM inactivation. Similarly, the effect of reduced PMS on the rate of inhibition of the *lac* carrier by NEM is completely abolished if the reaction is performed under argon instead of oxygen (H. R. Kaback, unpublished observations).

Experiments similar to those shown in Figure 1 have been performed at pH 6.6 in the presence of either 5 μM valinomycin or 0.25 μM nigericin, which selectively abolish the

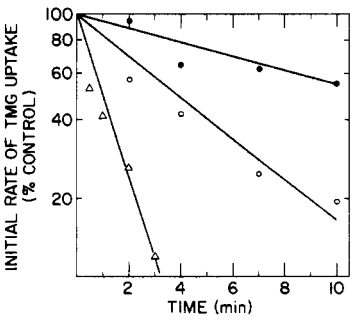

FIGURE 2: Inhibition of melibiose transport by NEM. Membrane vesicles of *E. coli* W3113-2 induced for melibiose transport were diluted to 1.0 mg of membrane protein/mL in 0.1 M potassium phosphate (pH 6.6). For inhibition in the absence of additions (○), NEM was added to a final concentration of 50 μM, and the mixture was incubated at room temperature for the indicated times, after which 20 volumes of buffer containing 0.1 M potassium phosphate (pH 6.6), 5 mM sodium sulfate, and 1.0 mM DTT was added to stop the reaction. The mixture was centrifuged at 40000*g* for 30 min, washed once, and resuspended in 0.1 M potassium phosphate (pH 6.6) and 5 mM sodium sulfate to a final protein concentration of 4 mg/mL. Initial rates of TMG uptake were then assayed at a protein concentration of 2 mg/mL in 50-μL final volume by addition of (in final concentrations) 10 mM magnesium sulfate, 20 mM potassium ascorbate (pH 6.6), 0.1 mM PMS, and after 30 s, 0.5 mM [^{14}C]TMG (15 mCi/mmol) as previously described (Cohn & Kaback, 1980). Inhibition in the presence of substrate (●) was carried out exactly as above except that the vesicles were preequilibrated for 20 min with 20 mM melibiose plus 5 mM sodium sulfate before the addition of 50 μM NEM. Inhibition by 50 μM NEM in the presence of reduced PMS (△) was also carried out in the same way, except that 20 mM potassium ascorbate and 10 μM PMS were added (under an oxygen atmosphere) 30 s before the addition of the NEM. All subsequent operations were as described above. Zero times of inhibition were carried out identically, except that 1 mM DTT was added before the NEM. The uninhibited rates of transport (100% activity) under the three conditions were the same and were 20 nmol of TMG min^{-1} (mg of protein)$^{-1}$.

Table III: Effect of Dinitrophenol on the Rate of Inhibition of the Melibiose Transporter by NEM[a]

	$t_{1/2}$ of inhibition (min)	
	−PMS	+PMS
−dinitrophenol	3.9	0.8
+dinitrophenol (5 mM)	3.6	3.6

[a] Membrane vesicles of *E. coli* W3113-2 induced for melibiose transport were diluted to 1.0 mg of membrane protein/mL in 0.1 M potassium phosphate (pH 6.6) and 10 mM magnesium sulfate (final concentrations) at room temperature. NEM was added to a final concentration of 50 μM in the presence or absence of 0.1 mM PMS plus 20 mM potassium ascorbate (pH 6.6) and dinitrophenol, as indicated. The incubation was terminated at various times after the addition of NEM by diluting 20-fold with buffer containing 0.1 M potassium phosphate (pH 6.6), 10 mM magnesium sulfate, 5 mM sodium sulfate, 5 mM dithiothreitol, and 1 mg/mL bovine serum albumin to facilitate the removal of dinitrophenol from the membrane. After centrifugation at 40000*g* for 30 min, the vesicles were washed once with buffer containing 0.1 M potassium phosphate, 10 mM magnesium sulfate, and 5 mM sodium sulfate and were finally resuspended in the same buffer to 2 mg of membrane protein/mL. Aliquots of 50 μL were then assayed for initial rates of TMG uptake by addition of 0.1 mM PMS, 20 mM potassium ascorbate (pH 6.6), and, after 30 s, 0.5 mM [^{14}C]TMG (15 mCi/mmol) as previously described (Cohn & Kaback, 1980). Control values were [in nmol min^{-1} (mg of protein)$^{-1}$] 27.0 and 25.3 for samples treated without and with reduced PMS, respectively, in the absence of DNP and 23.7 and 21.7 for samples treated without and with reduced PMS, respectively, in the presence of DNP.

membrane potential ($\Delta\Psi$) or the transmembrane pH gradient (ΔpH), respectively. As shown in Table IV, in both cases the rate at which NEM inactivates the lac carrier is accelerated

Table IV: Effect of Valinomycin and Nigericin on the Inhibition of Lactose Transport by NEM[a]

	$t_{1/2}$ of inhibition (min)	
	−PMS	+PMS
+5 μM valinomycin	2.9	1.1
+0.25 μM nigericin	3.2	0.8

[a] *E. coli* ML 308-225 membrane vesicles at pH 6.6, treated with the indicated concentrations of valinomycin or nigericin, were exposed to 0.5 mM NEM in the presence and absence of PMS plus ascorbate, following which initial rates of lactose uptake were determined as described in Figure 1. Rates of uptake before NEM treatment were 17 and 25 nmol of lactose min^{-1} (mg of protein)$^{-1}$ in the presence of valinomycin and nigericin, respectively.

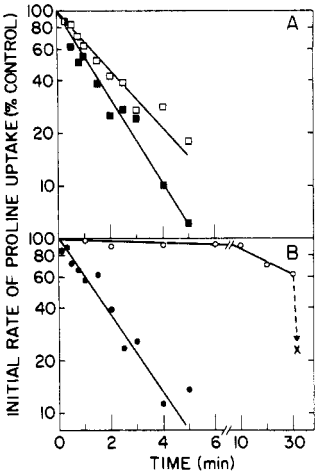

FIGURE 3: Time course of inhibition of proline transport by NNM (A) and GSMal (B) in the presence and absence of reduced PMS. (Panel A) *E. coli* ML 308-225 membrane vesicles were treated with 20 μM NNM (final concentration) in the presence (■) and absence (□) of PMS plus ascorbate exactly as described in Figure 1 for NEM. Following the inhibition reaction, active uptake of proline was determined as described under Materials and Methods and Figure 1. The final concentration of [U-^{14}C]proline was 13.6 μM (240 mCi/mmol). Uninhibited rates (100% activity) of proline uptake were 1.9 nmol min^{-1} (mg of protein)$^{-1}$. (Panel B) Vesicles were treated with 2.0 mM GSMal (final concentration) in the presence (●) and absence (○) of PMS plus ascorbate as described in Figure 1. Following inhibition for the times indicated, active uptake of proline was determined as above. The arrow and dotted line indicate a sample that was incubated with GSMal for 30 min in the absence of energy, following which PMS plus ascorbate was added for 1.5 min prior to the addition of DTT and the subsequent determination of proline uptake (×). Control vesicles to which DTT was added before GSMal lost no activity during a 30-min incubation at 26 °C.

by reduced PMS, demonstrating that either component of $\Delta\bar{\mu}_{H^+}$ ($\Delta\psi$ or ΔpH) is effective in this regard.

The proline carrier of *E. coli* is another transport system that is extremely sensitive to sulfhydryl reagents (Patel & Kaback, 1978). However, as shown in Figure 3A, the rate of inhibition of this system by NNM is only slightly enhanced by reduced PMS (1.2–1.5-fold stimulation in a number of experiments); with NEM, a similar small degree of enhancement is observed in the presence of reduced PMS (not shown). Strikingly, with the impermeant maleimide, GSmal, the rate of inhibition in the absence of reduced PMS is extremely slow and is increased over 30-fold in the presence of the electron donor (Figure 3B). The arrow and dotted line in Figure 3B indicate a sample to which PMS and ascorbate were added after 30-min incubation of the GSMal with vesicles. Incubation was continued for an addditional 1.5 min before DTT was added and the initial rate of proline uptake determined. Clearly, GSMal does not lose its inhibitory ac-

tivity during long preincubation with vesicles. These results are in marked contrast to the findings with the *lac* carrier where inhibition by each of the maleimides examined is enhanced to a similar extent by reduced PMS (Table I).

Discussion

The results presented here demonstrate that a number of transport systems in *E. coli* membrane vesicles exhibit increased sensitivity to maleimides in the presence of reduced PMS. Furthermore, the enhanced sensitivity is observed with both proton-linked (lactose and proline) and sodium-linked (melibiose) transport systems. Clearly, the effect is a consequence of the $\Delta\bar{\mu}_{H^+}$ generated by the oxidation of the electron donor since it is completely eliminated by the uncoupler dinitrophenol (Table III) or by carrying out the reaction under argon. Experiments using valinomycin or nigericin to selectively abolish one of the components of $\Delta\bar{\mu}_{H^+}$ ($\Delta\Psi$ or ΔpH, respectively) demonstrate that each component can independently bring about the increased sensitivity to maleimides (Table IV). It has recently been reported that the *lac* carrier of *E. coli* is inhibited by the histidine-specific reagent diethyl pyrocarbonate, that the rate of inhibition by this reagent is increased about 3-fold in the presence of reduced PMS, and that the effect is due to $\Delta\bar{\mu}_{H^+}$ (Padan et al., 1979). Similar effects of energization on sulfhydryl inhibition of the phosphate (LeQuoc et al., 1977) and adenosine nucleotide diphosphate (LeQuoc et al., 1979) translocators in mitochondria have been reported.

In the case of the *lac* carrier, it is apparent that although each of the maleimides examined inhibits active lactose uptake, the concentrations required to achieve a reasonable rate of inhibition vary considerably (Table I). It is unlikely that these differences are related to the inherent reactivities of the maleimides used since variation of the nitrogen substituent of the maleimides has little effect on reactivity (Karlin, 1969). Thus, the finding that the very hydrophobic maleimide NNM is effective at 20 μM, while the impermeant maleimide GSMal requires concentrations 100-fold greater would seem to indicate that the sensitive sulfhydryl group in the *lac* carrier is not freely accessible to the external medium. Whether it is buried in the membrane or exposed on the intravesicular surface is not clear from these observations. However, with this carrier, it is particularly noteworthy that the enhanced rate of inhibition induced by reduced PMS is essentially the same for all the maleimides examined (Table I). Thus, although the accessibility of the sulfhydryl group may be a factor in the inactivation reaction, this factor does not appear to change significantly upon energization. Otherwise, one would expect to observe more dramatic differences between the permeant and impermeant maleimides in the presence of reduced PMS. Rather, the results are consistent with the notion that energization brings about a change in the reactivity of the sensitive sulfhydryl group of the lactose carrier, perhaps via a conformational change in the protein.

Recent kinetic studies on the lactose carrier indicate that the carrier may function in two modes (Robertson et al., 1980). In one mode, which predominates under nonenergized conditions, the carrier catalyzes facilitated diffusion, a reaction characterized by a high apparent K_m (20 mM). Energization by either $\Delta\Psi$ or ΔpH causes the carrier to shift to a second mode in which it catalyzes active transport with a considerably lower apparent K_m (0.2 mM). The fraction of the population that shifts from the high to the low apparent K_m mode is a function of the magnitude of $\Delta\bar{\mu}_{H^+}$ to the second power. The present findings are consistent with these observations and support the suggestion that the shift between the two kinetic forms of the carrier may involve a structural or conformational change that alters the reactivity of a functionally required sulfhydryl group.

The rate at which NEM inhibits lactose uptake is dependent on the pH of the reaction (Table II), becoming more rapid as the pH is raised. This is due most likely to the fact that sulfhydryl groups become more nucleophilic at alkaline pH and are thus more susceptible to alkylation by maleimides. The observation that energization has almost no effect on the rate of inhibition of the lactose carrier by NEM at pH 5.5 may be related to the observation (Robertson et al., 1980) that the V_{max} for active lactose uptake is much lower at pH 5.5 than at pH 7.5, even though $\Delta\bar{\mu}_{H^+}$ at pH 5.5 exceeds that at pH 7.5. Again, this may be related to the conversion of the high apparent K_m form of the carrier to the low apparent K_m form in the presence of $\Delta\bar{\mu}_{H^+}$, a process that may occur to a much smaller extent at the acid pH.

The proline carrier, like the *lac* carrier, is susceptible to sulfhydryl inhibitors. In the absence of reduced PMS, NEM or NNM inhibits the proline carrier more rapidly than the lactose carrier (e.g., 20 μM NNM inhibits lactose uptake with a $t_{1/2}$ of 4 min and proline uptake with a $t_{1/2}$ of 1.6 min). Such differences clearly demonstrate that the inactivations observed occur at the level of the individual carriers and do not reflect a change in the general properties of the vesicles caused by the maleimides. When inactivation of proline transport by NNM is studied in the presence of reduced PMS, only a small stimulation of the rate is observed (Figure 3A). On the other hand, when GSMal is used to inhibit proline transport, striking effects are documented. In the absence of $\Delta\bar{\mu}_{H^+}$, GSMal inhibits proline transport extremely slowly (Figure 3B), while in the presence of reduced PMS, there is a dramatic stimulation of the inhibition rate (over 30-fold). Assuming that GSMal is relatively impermeant in this system, as has been demonstrated for other biological membranes (Abbott & Schachter, 1976), the large stimulation may reflect a $\Delta\bar{\mu}_{H^+}$-induced increase in the accessibility of the proline carrier to the external medium.

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Normal Mode Analysis of Lumiflavin and Interpretation of Resonance Raman Spectra of Flavoproteins†

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ABSTRACT: The normal modes of lumiflavin (10-methylisoalloxazine) are analyzed with a valence force field constructed with bond length–stretching force constant correlations and bending and interaction force constants transferred from small ring molecules. Observed resonance Raman (RR) bands of flavin are assigned to calculated modes on the basis of frequency and isotope shift matching. The normal mode patterns confirm previous inferences, based on selective effects of chemical substitutions, of localization to certain regions of the molecule. These results are used to interpret the observed variability of the prominent RR bands among different flavoproteins on the basis of protein–isoalloxazine interactions.

Resonance Raman (RR) spectroscopy is potentially useful for studying flavoprotein structure and dynamics. This technique allows in situ monitoring of vibrational modes of biological chromophores via the enhancement observed when the laser excitation source is tuned to the chromophore's electronic absorption band (Spiro & Loehr, 1975; Spiro & Stein, 1977). The flavin $\pi-\pi$ transitions (Sun et al., 1972; Eaton et al., 1975), occurring in the visible and near-ultraviolet region, are expected to provide enhancement of the in-plane modes of the isoalloxazine chromophore, whose structure is shown in Figure 1. Since this is the region of the coenzyme where the redox reactions involved in flavoenzyme reactivity are localized, the RR spectrum may reasonably be expected to probe biochemically significant structure changes.

The intense fluorescence exhibited by flavins has been a deterrent to RR studies. In 1977, however, flavin RR spectra were shown (Dutta et al., 1977) to be obtainable via coherent anti-Stokes Raman scattering (CARS), a technique for generating the Raman signal as a coherent beam of light, which can be filtered spatially from the isotropic fluorescence. In 1978 Nishina et al. showed that riboflavin binding protein (RBP) quenches the fluorescence of the bound riboflavin sufficiently to obtain good quality spontaneous RR spectra. These reports kindled interest in the field, and several additional flavin RR studies have appeared in the past two years (Dutta et al., 1978, 1980; Nishimura & Tsuboi, 1978; Kitagawa et al., 1979a,b; Beneckey et al., 1979; Dutta & Spiro, 1980; Schopfer & Morris, 1980; Benecky et al., 1980). Most of the available data are on oxidized flavin, although spectra of semiquinone forms (Dutta & Spiro, 1980) and of charge-transfer complexes (Kitagawa et al., 1976b) have also been obtained.

Attention has focused initially on the assignment of the observed RR bands to specific structural elements of the chromophore. A variety of chemically and isotopically substituted flavins have been examined, and qualitative interpretations of the resulting vibrational frequency shifts have been offered. These suggestions need to be extended, however, and to this end, we have carried out a normal mode analysis of the in-plant vibrations of lumiflavin, in which the ribose substituent at N_{10} is replaced by a methyl group (Figure 1). Bond length–stretching force constant correlations were used,

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